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(54) Title: DNA SEQUENCE OF A GENE OF HYDROXY-PHENYL PYRUVATE DIOXYGENASE AND PRODUCTION OF PLANTS CONTAINING A GENE OF HYDROXY-PHENYL PYRUVATE DIOXYGENASE AND WHICH ARE TOLERANT TO CERTAIN HERBICIDES

(54) Titre: SEQUENCE ADN D'UN GENE DE L'HYDROXY-PHENYL PYRUVATE DIOXYGENASE ET OBTENTION DE PLANTES CONTENANT UN GENE DE L'HYDROXY-PHENYL PYRUVATE DIOXYGENASE, TOLERANTES A CERTAINS HERBICIDES

(57) Abstract

DNA sequence of a gene of hydroxy-phenyl pyruvate dioxygenase and production of plants containing a gene of hydroxy-phenyl pyruvate dioxygenase and which are resistant to herbicides. DNA sequence of a gene of hydroxy-phenyl pyruvate dioxygenase; isolation from a bacteria or a plant; utilization for obtaining plants tolerant to herbicides.

(57) Abrégé

Séquence ADN d'un gène de l'hydroxy-phényl pyruvate dioxygénase et obtention de plantes contenant un gène de l'hydroxy-phényl pyruvate dioxygénase, résistantes aux herbicides. Séquence ADN d'un gène de l'hydroxy-phényl pyruvate dioxygénase; isolement à partir d'une bactérie ou d'une plante; utilisation pour l'obtention de plantes tolérantes aux herbicides.

DNA sequence of a hydroxyphenylpyruvate dioxygenase gene and obtainment of plants comprising a hydroxyphenylpyruvate dioxygenase gene, which are tolerant to certain herbicides

The present invention relates to a hydroxyphenylpyruvate dioxygenase (HPPD) gene, a chimeric gene comprising this gene as coding sequence and its use to obtain plants resistant to certain herbicides.

Certain herbicides have been disclosed, such as the isoxazoles described especially in the French Patent Applications 95 06800 and 95 13570 and especially isoxaflutole, a selective maize herbicide, diketonitriles such as those described in European Applications 0 496 630, 0 496 631, in particular 2-cyano-3-cyclopropyl-1-(2-SO_2CH_3-4-CF_3-phenyl)propane-1,3-dione and 2-cyano-3-cyclopropyl-1-(2-SO_2CH_3-4-2,3Cl_2phenyl)propane-1,3-dione, triketones described in European Applications 0 625 505 and 0 625 508, in particular sulcotrione. However, a tolerance gene to such herbicides has not been described.

Hydroxyphenylpyruvate dioxygenase is an enzyme which catalyses the conversion reaction of para-hydroxyphenylpyruvate into homogentisate.

In addition, the amino-acid sequence of hydroxyphenylpyruvate dioxygenase from *Pseudomonas sp.* P.J. 874 has been described, without there being a

description of its role in the tolerance of the plants to herbicides (Rüetschi et al.: Eur. J. Biochem. 205, 459-466, 1992). This document does not give a description of the gene coding for this protein.

There have now been discovered the sequence of a gene of this type and that such a sequence could, once incorporated into plant cells, produce an over-expression or an activation of HPPD in the plants giving to the latter a worthwhile tolerance to certain novel herbicides, such as those of the isoxazoles family or that of the triketones.

The present invention accordingly provides an isolated sequence of a gene expressing a hydroxyphenylpyruvate dioxygenase (HPPD), characterized in that it is from *Pseudomonas fluorescens*.

The invention also provides an isolated sequence expressing a HPPD, characterized in that it is of plant origin, such as especially of monocotyledonous or dicotyledonous plants, especially of *Arabidopsis* or of Umbelliferae, such as, for example, the carrot (*Daucus carotta*). It can be native or wild or possibly mutated while at the same time fundamentally retaining a property of herbicidal tolerance against HPPD inhibitors, such as herbicides of the isoxazoles family or that of the triketones. The sequences shown in SEQ ID NO:2 and SEQ ID NO:3 are preferred.

The invention likewise relates to a process of isolating the above gene, characterized in that:

- as primers, some oligonucleotides from the amino-acid sequence of an HPPD are chosen,
- starting from these primers, amplification fragments are synthesized by PCR
- the gene is isolated by creation and screening of a genomic bank and
- the gene is cloned.

Preferably, primers from the HPPD sequence of a bacterium of the genus *Pseudomonas* are used. Particularly preferably, they are from *Pseudomonas fluorescens*.

The invention also relates to the use of a gene coding for HPPD in a process for the transformation of plants, as a marker gene or as a coding sequence allowing tolerance to certain herbicides to be conferred on the plant. It can likewise be used, in association with other marker genes and/or coding sequence, for an agronomic property.

The coding gene can be of any origin, native or wild or possibly mutated, while at the same time fundamentally retaining a property of herbicidal tolerance against inhibitors of HPPD, such as herbicides of the isoxazoles family or that of the triketones. As coding sequence, especially that according to the invention such as described above, can

be used.

The transformation of plant cells can be achieved by any appropriate known means. A series of methods consists in bombarding cells or protoplasts with particles to which are coupled the DNA sequences.

Another series of methods consists in using, as means of transfer into the plant, a chimeric gene inserted into a Ti plasmid of *Agrobacterium tumefaciens* or Ri plasmid of *Agrobacterium rhizogenes*.

An object of the present invention is also a chimeric gene for the genetic transformation of plants comprising, in the transcription direction, at least one promoter regulation sequence from a gene expressed naturally in plants, a heterologous coding sequence (for example of bacterial, such as *Pseudomonas sp.* or of plant origin) which expresses hydroxyphenylpyruvate dioxygenase and at least one polyadenylation sequence.

The promoter regulation sequence used can be any promoter sequence of a gene which is naturally expressed in plants, in particular a promoter of bacterial, viral or plant origin, such as, for example, that of a gene of the small subunit of ribulose biscarboxylase (RuBisCO) or that of a gene of α-tubulin (European Application EP No. 0 652 286), or alternatively of a plant virus gene such as, for example, that of cauliflower mosaic virus (CAMV 19S or

35S), but any suitable promoter can be used. Preferably, recourse is made to a promoter regulation sequence which favours the overexpression of the coding sequence, such as, for example, that comprising at

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least one histone promoter such as described in European Application EP 0507698.

According to the invention, it is equally possible to use, in association with the promoter regulation sequence, other regulation sequences which are situated between the promoter and the coding sequence, such as "enhancer" transcription activators, such as, for example, tobacco etch virus (TEV) translation activator described in the Application WO87/07644, or of transit peptides, either single, or double, and in this case possibly separated by an intermediate sequence, that is to say comprising, in the transcription direction, a sequence coding for a transit peptide of a plant gene coding for a plastid localization enzyme, a part of the sequence of the N-terminal mature part of a plant gene coding for a plastid localization enzyme, then a sequence coding for a second transit peptide of a plant gene coding for a plastid localization enzyme, formed by a part of the sequence of the N-terminal mature part of a plant gene coding for a plastid localization enzyme, such as described in European Application No. 0 508 909. The invention provides a chimeric gene which comprises, between the promoter regulation sequence and the coding sequence, an optimized transit peptide comprising, in the transcription sense, a sequence coding for a transit peptide of a plant gene coding for an enzyme with plastid localization, a part of the sequence of the N-terminal mature part of a plant gene coding for an enzyme

with plastid localization, then a sequence coding for a second transit peptide of a plant gene coding for an enzyme with plastid localization.

It is possible to use as terminator or polyadenylation regulation sequence any corresponding sequence of bacterial origin, such as, for example, the nos terminator of *Agrobacterium tumefaciens*, or even of plant origin, such as, for example, a histone terminator such as described in European Application

EP No. 0 633 317.

An object of the present invention is also plant cells, of monocotyledonous or dicotyledonous plants, especially of crops, transformed according to one of the processes described above and comprising in their genome an efficacious quantity of a gene expressing hydroxyphenylpyruvate dioxygenase (HPPD). The invention also provides a plant regenerated from such transformed plant cells. It has been observed that plants transformed in this way have a significant tolerance to certain novel herbicides such as the isoxazoles described especially in French Patent Applications 9506800 and 95 13570 and especially of 4-[4-CF3-2-(methylsulphonyl)benzoyl]-5-cyclopropylisoxazole, and especially isoxaflutole, a selective maize herbicide, the diketonitriles such as those described in European Applications 0 496 630, 0 496 631, in particular 2-cyano-3-cyclopropyl-1-(2-SO_2CH_3-4-CF_3-phenyl)propane-1,3-dione and 2-cyano-3-cyclopropyl-1-(2-SO_2CH_3-4-2,3-Cl_2-phenyl)propane-1,3-dione, the triketones described in European Applications 0 625 505 and 0 625 508, in particular sulcotrione.

Finally, an object of the invention is a method of weeding plants, especially crops, with the aid of a herbicide of this type, characterized in that this herbicide is applied to plants transformed according to the invention, both pre-sowing, pre-emergence and post-emergence of the crop.

An object of the invention is also the use of

the HPPD gene as a marker gene in the course of the "transformation-regeneration" cycle of a plant species and selection on the above herbicide.

The different aspects of the invention will be better understood with the aid of the experimental examples below.

Example 1: Isolation of the HPPD gene of *P. fluorescens* A32

Starting from the amino-acid sequence of HPPD of *Pseudomonas sp.* P.J. 874 (published by Rüetschi U. et al., 1992, Eur. J. Biochem. 205: 459-466), the sequence of different oligonucleotides is deduced in order to amplify by PCR a part of the coding sequence of HPPD of *P. fluorescens* A 32 (isolated by McKellar, R.C. 1982, J. Appl. Bacteriol., 53: 305-316). An amplification fragment of the gene of this HPPD has been used to screen a partial genomic bank of *P. fluorescens* A32 and thus to isolate the gene coding for this enzyme.

A) Preparation of genomic DNA of *P. fluorescens* A32

The bacteria was cultivated in 40 ml of M63 minimum medium (KH_2PO_4 13.6 g/l, $(NH_4)_2SO_4$ 2 g/l, $MgSO_4$ 0.2 g/l, $FeSO_4$ 0.005 g/l, pH 7 plus L-tyrosine 10 mM as the sole carbon source) at 28°C for 48 hours.

After washing, the cells are taken up in 1 ml of lysis buffer (100 mM tris HCl, pH 8.3, 1.4 M NaCl and 10 mM EDTA) and incubated for 10 minutes at 65°C.

After a phenol/chloroform treatment (24:1) and a chloroform treatment, the nucleic acids are precipitated by addition of one volume of isopropanol, then taken up in 300 µl of sterile water and treated with final 10 µg/ml RNAse. The DNA is treated afresh with phenol/chloroform, chloroform and reprecipitated by addition of 1/10 of the volume of 3 M sodium acetate, pH 5 and 2 volumes of ethanol. The DNA is then taken up in sterile water and determined.

B) Choice of the oligonucleotides and syntheses

Starting from the amino-acid sequence of HPPD of *Pseudomonas sp.* P.J. 874, five oligonucleotides are chosen, two directed in the terminal NH_2 direction of the protein towards the COOH terminal of the protein and three directed in the opposite direction (see Fig. 1). The choice was dictated by the two following rules:

- a stable 3' end of the oligonucleotide, that is to say at least two bases without ambiguity.
- the smallest degeneracy possible.

The oligonucleotides chosen have the following sequences:

P1: 5'TA(C/T)GA(G/A)AA(C/T)CCIATGGG3'

P2: 5'GA(G/A)ACIGGICCIATGGA3'

P3: 5'AA(C/T)TGCATIA(G/A)(G/A)AA(C/T)TC(C/T)TC3'

P4: 5'AAIGCIAC(G/A)TG(C/T)TG(T/G/A)ATICC3'

P5: 5'GC(C/T)TT(A/G)AA(A/G)TTICC(C/T)TCIC3'

They were synthesized on a Cyclone plus DNA synthesizer of the make MILLIPORE.

With these five oligonucleotides, the amplification fragments which must be obtained theoretically by PCR starting from the sequence SEQ ID No. 1 have the following sizes:

with the primers P1 and P3 -------> approximately 690 bp

with the primers P1 and P4 -------> approximately 720 bp

with the primers P1 and P5 --------> approximately 1000 bp

with the primers P2 and P3 -------> approximately 390 bp

with the primers P2 and P4 --------> approximately 420 bp

with the primers P2 and P5 --------> approximately 700 bp

C) Amplification of a coding part of HPPD of *P. fluorescens* A32.

The amplifications were carried out on a PERKIN ELMER 9600 PCR apparatus and with PERKIN ELMER Taq polymerase with its buffer under standard conditions, that is to say for 50 µl of reaction mixture there are dNTP at 200 µM, primers at 20 µM, 2.5 units of Taq polymerase and 2.5 µg of DNA of *P. fluorescens* A32.

The amplification programme used is 5 min at 95°C and then 35 <45 sec 95°C, 45 sec 49°C, 1 min 72°C> cycles followed by 5 min at 72°C.

Under these conditions, all the amplification fragments obtained have a size compatible with the theoretical sizes given above, which is a good indication of the specificity of the amplifications.

The amplification fragments obtained with the sets of primers P1/P4, P1/P5 and P2/P4 are ligated into pBSII SK(-) after digestion of this plasmid by Eco RV and treatment with the terminal transferase in the presence of ddTTP as described in HOLTON T.A. and GRAHAM M.W. 1991, N.A.R., Vol. 19, No. 5, p. 1156.

A clone of each of the three types is partially sequenced; this allows it to be confirmed that a part of the coding region of the HPPD of *P. fluorescens* A32 has indeed been amplified in the three cases. The P1/P4 fragment is retained as probe in order to screen a partial genomic bank of *P. fluorescens* A32 and to isolate the complete gene of the HPPD.

D) Isolation of the gene

By Southern it is shown that a 7 Kbp fragment hybridizes, after digestion of the DNA of *P. fluorescens* A32 by the restriction enzyme BamHI, with the probe HPPD P1/P4. 400 µg of DNA of *P. fluorescens* A32 are thus digested with the restriction enzyme BamHI and the DNA fragments making up approximately 7 Kbp are

purified on agarose gel.

These fragments are ligated into pBSII SK(-), the latter is digested with BamHI and dephosphorylated by treatment with alkaline phosphatase. After transformation in *E. coli* DH10b, the partial genomic bank is screened with the probe HPPD P1/P4.

A positive clone was isolated and called pRP A. Its simplified map is given in Figure 2. On this map is indicated the position of the coding part of the HPPD gene. It is composed of 1077 nucleotides which code for 358 amino acids (see SEQ ID No. 1). The HPPD of *P. fluorescens* A32 has a good amino-acid homology with that of *Pseudomonas* sp. strain P.J. 874, in fact there is 92 % agreement between these two proteins (see Fig. 3).

Example 2: Construction of two chimeric genes

To confer plant tolerance to herbicides inhibiting HPPD, two chimeric genes are constructed:

The first consists in putting the coding part of the HPPD gene of *P. fluorescens* A32 under the control of the double histone promoter (European Patent No. 0 507 698) followed by tobacco etch virus translational enhancer (TEV) (pRTL-GUS (Carrington and Freed, 1990; J. Virol. 64: 1590-1597)) with the terminator of the nopaline synthase gene. The HPPD will then be localized in the cytoplasm.

The second will be identical to the first, except that the optimized transit peptide (OTP) is

intercalated between the TEV transcription activator and the coding part of the HPPD (European Application EP No. 0 508 909). The HPPD will then be localized in the chloroplast.

A) Construction of the vector pRPA-RD-153:

- pRDA-RD-11 A derivative of pBS-II SK(-) (Stratagene catalog #212206) containing the polyadenylation site of nopaline synthase (NOS polyA) (European Application EP No. 0 652 286) is cloned between the *KpnI* and *SalI* sites. The *KpmI* site is transformed into a NotI site by treatment with T4 DNA polymerase I in the presence of 150 μM of deoxynucleotide triphosphates and then ligation with an NotI linker (Stratagene catalog #1029). An NOS polyA cloning cassette is thus obtained.

- pRPA-RD-127: A derivative of pRPA-BL-466 (European Application EP No. 0 337 899) cloned in pRPA-RD-11 creating an expression cassette of the *oxy* gene and containing the promoter of the small subunit of ribulose biscarboxylase:

"promoter (SSU) - *oxy* gene - NOS polyA"

To create this plasmid, pRPA-BL-488 was digested with XbaI and HindIII to isolate a fragment of 1.9 kbp comprising the SSU promoter and the *oxy* gene which was ligated into the plasmid pRPA-RD-11, digested with compatible enzymes.

- pRPA-RD-132: This is a derivative of pRPA-BL-488 (European Application EP No. 0 507 698) cloned

into pRPA-RD-127 with creation of an expression cassette of the *oxy* gene with the double histone promoter:

"double histone promoter - *oxy* gene - NOS polyA"

To produce this plasmid, pRPA-BL-466 is digested with HindIII, treated with Klenow and then redigested with NcoI. The purified fragment of 1.35 kbp containing the histone double promoter H3A748 is ligated with the plasmid pRPA-RD-127 which had been digested with XbaI, treated with Klenow and redigested with NcoI.

- pRPA-RD-153: This is a derivative of pRPA-RD-132 containing the translation activator of the tobacco etch virus (TEV). pRTL-GUS (Carrington and Freed, 1990; J. Virol. 64: 1590-1597) is digested with *NcoI* and *EcoRI* and the 150 bp fragment is ligated into pRPA-RD-132 digested with the same enzymes. An expression cassette containing the promoter : "double histone promoter - TEV - *oxy* gene - NOS polyA" is thus created.

B) Construction of the vector pRPA-RD-185:

pUC19/GECA: A derivative of pUC-19 (Gibco catalog #15364-011) containing numerous cloning sites. pUC-19 is digested with *EcoRI* and ligated with the oligonucleotide linker 1:

Linker 1: AATTGGGCCA GTCAGGCCGT TTAAACCCTA GGGGGCCCG
CCCGGT CAGTCCGGCA AATTTGGGAT CCCCCGGGC TTAA

The selected clone contains an *EcoRI* site

followed by the polylinker which contains the following sites: *EcoRI, ApaI, AvrII, PmeI, SfiI, SacI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI* and *HindIII*.

pRPA-RD-185: this is a derivative of pUC19/GECA containing a modified polylinker. pUC19/GECA is digested with HindIII and ligated with the oligonucleotide linker 2:

Linker 2: AGCTTTTAAT TAAGGCGCGC CCTCGAGCCT GGTTCAGGG
AAATTA ATTCCGCGCG GGAGCTCGGA CCAAGTCCC TCGA

The selected clone contains a *HindIII* site in the centre of the polylinker which now contains the following sites: *EcoRI, ApaI, AvrII, PmeI, SfiI, SacI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI, HindIII, PacI, AscI, XhoI* and *EcoNI*.

C) Construction of the vector pRP T:

- pRP O: a derivative of pRPA-RD-153 containing an expression cassette of HPPD, double histone promoter - TEV - HPPD gene - terminator Nos. To produce pRP O, pRPA-RD153 is digested with HindIII, treated with Klenow and then redigested with NcoI to remove the *oxy* gene and replace it by the HPPD gene coming from the pRP A plasmid by BstEII digestion, Klenow treatment and redigestion with NcoI.

- pRP R: to obtain the plasmid, pRP O was digested with PvuII and SacI, the chimeric gene was purified and then ligated into pRPA-RD-185 and the latter was digested with PvuII and SacI.

- pRP T: was obtained by ligation of the

chimeric gene coming from pRP R after digestion with SacI and HindIII in the plasmid pRPA-BL 150 alpha2 digested with the same enzymes (European Application EP No. 0 508 909).

The chimeric gene of the pRP T vector thus has the following structure:

Double histone promoter	TEV	Coding region of HPPD	nos terminator

D) Construction of the pRP V vector

- pRP P: this is a derivative of pRPA-RD-7 (European Application EP No. 0 652 286) containing the optimized transit peptide followed by the HPPD gene. It was obtained by ligation of the coding part of HPPD coming from pRP A by BstEII and NcoI digestion, Klenow treatment and from the plasmid pRPA-RD-7, the latter digested with SphI and AccI and treated with DNAse polymerase T4.

- pRP Q: a derivative of pRPA-RD-153 containing an expression cassette of HPPD, double histone promoter - TEV - OTP - HPPD gene - Nos terminator. To construct it, the plasmid pRPA-RD-153 is digested with SalI, treated with Klenow and then redigested with NcoI to remove the *oxy* gene and replace it by the HPPD gene released from the pRP P plasmid by BstEII digestion, Klenow treatment and redigestion with NcoI.

- pRP S: to obtain it, the plasmid pRP Q was

digested with PvuII and SacI to release the chimeric gene, which was ligated into pRPA-RD-185, the latter digested with PvuII and SacI.

- pRP V: it was obtained by ligation of the chimeric gene released from pRP S, after digestion with SacI and HindIII, into the plasmid pRPA-BL 150 alpha2 (European Application EP No. 0 508 909).

The chimeric gene of the pRP Q vector thus has the following structure:

Double histone promoter	TEV	OTP	Coding region of HPPD	nos terminator

Example 3: Transformation of the industrial tobacco PBD6

In order to determine the efficacy of these two chimeric genes, these were transferred to industrial tobacco PBD6 according to the transformation and regeneration procedures already described in European Application EP No. 0 508 909.

1) Transformation:

The vector is introduced into the non-oncogenic strain of *Agrobacterium* EHA 101 (Hood et al., 1987) which carries the cosmid pTVK 291 (Komari et al., 1986). The transformation technique is based on the procedure of Horsh R. et al. (1985), Science, 227, 1229-1231.

2) Regeneration:

The regeneration of the tobacco PBD6 (origin

SEITA France) from foliar explants is carried out on a Murashige and Skoog (MS) base medium comprising 30 g/l of sucrose as well as 200 ug/ml of kanamycin. The foliar explants are selected on plants in the greenhouse or in vitro and transformed according to the foliar discs technique (Science 1985, Vol. 227, p. 1229-1231) in three successive steps: the first comprises the induction of shoots on an MS medium to which is added 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 15 days. The shoots formed during this step are then developed by culture on an MS medium to which is added 30 g/l of sucrose, but not containing any hormone, for 10 days. Developed shoots are then selected and cultured on an MS rooting medium of half salts, vitamins and sugars content and not containing any hormone. At the end of approximately 15 days, the rooted shoots are placed in earth.

Example 4: Measurement of the tolerance of the tobacco to 4-[4-CF_3-2-(methylsulphonyl)benzoyl]-5-cyclopropylisoxazole: post-emergence treatment

On leaving in-vitro culture, the transformed tobacco plantlets were acclimatized in a greenhouse (60 % relative humidity; temperature: 20°C during the night and 23°C during the day) for five weeks and then treated with 4-[4-CF_3-2-(methylsulphonyl)benzoyl]-5-cyclopropylisoxazole.

The control tobacco, non-transformed and

treated with 4-[4-CF_3-2-(methylsulphonyl)benzoyl]-5-cyclopropylisoxazole in doses ranging from 50 to 400 g/ha, develops chloroses in approximately 72 hours, which intensify to develop into very pronounced necroses in a week (covering approximately 80 % of the terminal leaves).

After transformation, this same tobacco, which overexpresses the HPPD of *P. fluorescens*, is very well protected against treatment with 4-[4-CF_3-2-(methylsulphonyl)benzoyl]-5-cyclopropylisoxazole at a dose of 400 g/ha.

If the overexpressed enzyme is in the cytoplasm, that is to say if the transformation was carried out with the gene carried by the vector pRP T, then the plant shows very slight chloroses which are all localized on the intermediate leaves.

If the overexpressed enzyme is in the chloroplast, that is to say if the transformation was carried out with the gene carried by the vector pRP V, then the plant is perfectly protected and does not show any symptoms.

Example 5: Measurement of the tolerance of the tobacco to 4-[4-CF3-2-(methylsulphoyl)benzoyl]-5-cyclopropylisoxazole: pre-emergence treatment

a) in vitro test:

Tobacco seeds harvested from plants from the "transformation-regeneration" cycle and resistant to isoxaflutole foliar treatment are used at a dose of

400 g/h described in Examples 1 to 3.

These seeds were sown in boxes containing plant agar at 10 g/l and isoxaflutole at different concentrations ranging from 0 to 1 mg/l. Germination was then carried out at 25°C with a photoperiod of 12 hours of light/12 hours of darkness.

According to this protocol, wild tobacco seeds were germinated as well as seeds of the two types of transgenic tobacco, that is to say CY tobaccos, with localization of the HPPD in the cytoplasm, and the CO tobaccos with localization of the HPPD in chloroplast.

Resistance measurements are carried out visually between 2 and 3 weeks after sowing.

The results are recorded in the table below.

isoxaflutole concentration	Wild tobacco	CY tobacco	CO tobacco
0 mg/l	100 % of the seeds germinate without symptoms°	100 % of the seeds germinate without symptoms°	100 % of the seeds germinate without symptoms
0.05 mg/l	20 % of the seeds germinate and show symptoms°	75 % of the seeds germinate* without symptoms°	75 % of the seeds germinate* without symptoms°

0.1 mg/l	no germination	75 % of the seeds germinate* without symptoms°	75 % of the seeds germinate* without symptoms°
0.5 mg/l	no germination	75 % of the seeds germinate* without symptoms°	75 % of the seeds germinate* without symptoms°
1 mg/l	no germination	75 % of the seeds germinate* with slight symptoms°	75 % of the seeds germinate* without symptoms°

° the symptoms which the plantlets show in the course of germination are more or less significant deformations of the cotyledons and above all a bleaching of the tissues which are normally photosynthetic and thus green.

* 75 % of the seeds germinate because seeds from the self-fertilization of single-locus plants coming from the "transformation-regeneration" cycle and thus only carrying the tolerance gene on one chromosome were sown.

Working in the same way with the following products,

Product No. 51 of American Patent 4 780 127, the same results are obtained at a concentration of 0 mg/l and 0.1 mg/l on wild tobacco and CO tobacco.

b) greenhouse test:

Measurement is carried out as in Example 4, apart from the treatment being carried out pre-emergence, 24 hours before sowing. Wild sowing is carried out normally. Under these conditions, it is observed that, for the non-treated control sowings, there is no germination for any dose of herbicide at least equal to 10 g/ha. On the contrary, the CY tobaccos do not show any symptoms, such as defined in paragraph a), up to and including 100 g/ha. Similarly, the CO tobaccos do not show any symptoms, such as defined in paragraph a), up to and including 200 g/ha.

These results show clearly that the HPPD gene of *P. fluorescens* confers a tolerance to the tobacco against pre-emergence treatments with isoxaflutole. This tolerance is better if the protein is localized in the chloroplast in place of the cytoplasm.

Example 6:

With the aim of studying whether the HPPD gene of *Pseudomonas fluorescens* can be used as a marker gene in the course of the "transformation-regeneration" cycle of a plant species, tobacco was transformed with the HPPD gene and transformed plants were obtained after selection on isoxaflutole.

Material and methods and results

The chimeric gene pRP V described below is transferred into industrial tobacco PBD6 according to the transformation and regeneration procedures already described in European Application EP No. 0 508 909.

The chimeric gene of the vector pRP V has the following structure:

Double histone promoter	TEV	OTP	Coding region of HPPD	nos terminator

1) Transformation:

The vector is introduced into the *Agrobacterium* EHA 101 non-oncogenic strain (Hood et al., 1987) which carries the cosmid pTVK 291 (Komari et al., 1986). The transformation technique is based on the procedure of Horsh et al. (1985).

2) Regeneration:

The regeneration of the tobacco PBD6 (origin SEITA France) from foliar explants is carried out on a Murashige and Skoog (MS) base medium comprising 30 g/l of sucrose as well as 350 mg/l of cefotaxime and 1 mg/l of isoxaflutole. The foliar explants are selected on plants in a greenhouse or in vitro and transformed according to the foliar discs technique (Science 1985, Vol. 227, p. 1229-1231) in three successive steps: the first comprises the induction of shoots on an MS medium to which is added 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 15 days and 1 mg/l of

isoxaflutole. The green shoots formed in the course of this step are then developed by culture on an MS medium to which are added 30 g/l of sucrose and 1 mg/l of isoxaflutole, but not containing hormone, for 10 days. Developed shoots are then selected and are cultured on an MS rooting medium of half salts, vitamins and sugars content and 1 mg/l of isoxaflutole and not containing any hormone. At the end of approximately 15 days, the rooted shoots are placed in earth.

All the plantlets obtained according to this protocol are analysed by PCR with specific primers of the HPPD of *P. fluorescens*. This PCR analysis has enabled it to be confirmed that all the plantlets thus obtained have indeed integrated the HPPD gene.

In conclusion, this assay confirms that the HPPD gene can be used as marker gene and that, associated with this gene, isoxaflutole can be a good selection agent.

Examples 7 and 8: Isolation of the HPPD gene of *Arabidopsis thaliana* and of the HPPD gene of carrot (*Daucus carotta*)

a) Construction of cDNA banks

mRNAs extracted from young plantlets of *Arabidopsis thaliana* and mRNAs extracted from carrot cells in culture served to construct two cDNA banks in the vector Uni Zap™ XR marketed by the company Stratagen, following the protocol recommended by this company.

b) Screening of the cDNA banks

These two banks were screened with the aid of a probe corresponding to a cDNA of *Arabidopsis thaliana* of partial length, obtained via the Arabidopsis Biological Resource Center (Ohio, USA) and indexed: EST clone No. 91B13T7. This clone is formed of approximately 500 base pairs of which only 228 had been sequenced by the MSU-DOE Plant Research Laboratory in the context of random sequencing of cDNA of *Arabidopsis thaliana*. We completely sequenced the 500 base pairs before using this clone to screen our cDNA banks of *Arabidopsis thaliana* and of carrot with the aid of the classical technique of hybridization of lysis regions (*reference ?*).

c) A cDNA of *Arabidopsis thaliana* (SEQ ID No. 2) corresponding to 1338 base pairs was obtained. This cDNA has a translation initiation start codon in position 25 and a translation end codon in position 1336. This cDNA is thus complete and codes for a protein of 445 amino acids.

d) A cDNA of carrot (*Daucus carotta*) (SEQ ID No. 3) corresponding to 1329 base pairs was obtained. This cDNA has a translation initiation start codon in position 1 and a translation finish codon in position 1329. This cDNA is thus complete and codes for a protein of 442 amino acids.

The sequences illustrated are the following:

SEQ ID No. 1 Sequence of the HPPD gene of *Pseudomonas fluorescens* A32

SEQ ID No. 2

cDNA sequence of HPPD of *Arabidopsis thaliana*

SEQ ID No. 3

cDNA sequence of HPPD of *Daucus carotta*

The figures below are given by way of indication to illustrate the invention.

Figure 1 represents the protein sequence of the HPPD of *Pseudomonas sp.* strain P.J 874 and the theoretical nucleotide sequence of the corresponding coding part; the five oligonucleotides chosen to carry out the amplification of a part of this coding region are symbolized by the five arrows.

Figure 2 represents the mapping of the plasmid with the genomic DNA fragment of 7 kb comprising the gene of the HPPD of *P. fluorescens* A32.

Figure 3 gives the comparison of the amino-acid sequences of the HPPD of *P fluorescens A32* and of the HPPD of *Pseudomonas sp.* strain P.J.874 (only the divergent amino acids between the two sequences are indicated) as well as the concensus sequence.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Sequence listing

(1) GENERAL INFORMATION

(i) APPLICANT: Sailland, Alain
Rolland, Anne
Matringe, Michel
Pallett, Kenneth E

(ii) TITLE OF INVENTION: DNA SEQUENCE OF A HYDROXYPHENYLPYRUVATE DIOXYGENASE GENE AND OBTAINMENT OF PLANTS COMPRISING THIS HYDROXYPHENYLPYRUVATE DIOXYGENASE GENE, WHICH ARE RESISTANT TO CERTAIN HERBICIDES

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Francois Chretien
(B) STREET: 14-20 rue Pierre BAIZET
(C) CITY: Lyon Cedex 09
(E) COUNTRY: France
(F) ZIP: 69263

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Patentin Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: FR PH95033
(B) FILING DATE: 02-JUN-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Chretien, Francois

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 72-29-26-42
(B) TELEFAX: 72-29-28-43

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1077 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pseudomonas fluorescens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1.

```
ATCGGTAGATC TATACGAAAA CTCAATGGGC CTGATGGGCT TTGAATTCAT CGAATTAGCG      60
[illegible]                                                             120
[illegible]                                                             180
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GCCGGCGCGC CGTTGTACCT GATCGACCGT TTCGGCGAAG GCAGCTCGAT CTACGACATC      420
GACTTCGTGT ACCTCGAAGG TGTGGAGCGC AATCCGGTCG GTGCAGGTCT CAAAGTCATC      480
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CACGTGGCGT TCCTCACCGA CGACCTGGTC AAGACCTGGG ACGCGTTGAA GAAAATCGGC      780
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GACCACGGCG AGCCGGTGGA TCAACTGCAG GCACGCGGTA TCCTGCTGGA CGGATCTTCC      900
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TCTTCGAAT TCATCCAGCG CAAGGGCGAC GATGGGTTTG GCGAGGGCAA CTTCAAGGCG     1020
CTGTTCGAGT CCATCGAACG TGACCAGGTG CGTCGTGGTG TATTGACCGC CGATTAA        1077
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1338 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Arabidopsis thaliana
(B) STRAIN: Columbia
(D) DEVELOPMENTAL STAGE: Young green plant

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Uni zap XR STRATAGENE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
ATGGGCCACC AAAACGCCGC CGTTTCAGAG AATCAAAACC ATGATGACGG CGCTGCGTCG       60
TCGCCGGGAT TCAAGCTCGT CGGATTTTCC AAGTTCGTAA GAAAGAATCC AAAGTCTGAT      120
```

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TCAAACATCG TTCACGTCTC TTACCTACTC ACCTCCGGTG ACCTCCGATC GCTTTTCACT 300

ATTCGTTACT GTCCGTCTCT GTCCGCCGGA GAGATTAAAC CGACAACCAC AGCTTCTATC 360

TCAAGTTTCG ATCATGGCTC TTGTCGTTCC TTCTTCTCTT CACATGGTCT CGGTGTTAGA 420

GCCGTTGCGA TTGAAGTAGA AGACGCAGAG TCAGCTTTCT CCATCAGTGT AGCTAATGGC 480

GCTATTCGTT CGTCGCCTCC TATCGTCCTC AATGAAGCAG TTACGATCGC TGAGGTTAAA 540

CTATACGGCG ATGTTGTTCT CCGATATGTT AGTTACAAAG CAGAAGATAC CGAAAAATCC 600

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ATCCGGCGGC TTGACCACGC CGTGGGAAAC GTTCCTCAGC TTGGTCCGGC TTTAACTTAT 720

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GCCGAGAGCG GTTTAAATTC AGCGGTCCTG GCTAGCAATG ATGAAATGGT TCTTCTACCG 840

ATTAACGAGC CAGTGCACGG AACAAAGAGG AAGAGTCAGA TTCAGACGTA TTTGGAACAT 900

AACGAAGGCG CAGGGCTACA ACATCTGGCT CTGATGAGTG AAGACATATT CAGGACCCTG 960

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TTCACAAAAC CACTAGGTGA CAGGCCGACG ATATTTATAG AGATAATCCA GAGAGTAGGA 1200

TGCATGATGA AAGATGAGGA AGGGAAGGCT TACCAGAGTG GAGGATGTGG TGGTTTTGGC 1260

AAAGGCAATT TCTCTGAGCT CTTCAAGTCC ATTGAAGAAT ACGAAAAGAC TCTTGAAGCC 1320

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1329 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Daucus carota
(D) DEVELOPMENTAL STAGE: Suspension cells

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Uni zap XR STRATAGENE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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```
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GCTGCATGA                                                          1329
```

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Isolated sequence of a gene expressing a hydroxyphenylpyruvate dioxygenase (HPPD) for producing transgenic plants that are tolerant to certain herbicides, characterised in that said sequence is obtainable from *Pseudomonas fluorescens* and comprises the sequence set forth in SEQ ID NO:1.

2. Isolated sequence of a gene expressing a hydroxyphenylpyruvate dioxygenase (HPPD) for producing transgenic plants that are tolerant to certain herbicides, characterised in that said sequence is of plant origin.

3. Sequence according to claim 2, characterized in that it is from *Arabidopsis*.

4. Sequence according to claim 3, comprising the sequence set forth in SEQ ID NO:2.

5. Sequence according to claim 2, characterized in that it is from an umbelliferous plant.

6. Sequence according to claim 5, wherein the umbelliferous plant is a carrot.

7. Sequence according to claim 6, comprising the sequence set forth in SEQ ID NO:3.

8. Chimeric gene for the genetic transformation of plants comprising, in the transcription sense:

- at least one promoter regulation sequence from a gene expressed naturally in plants,
- a heterologous coding sequence,
- at least one polyadenylation sequence, characterized in that the coding sequence is a sequence of a gene which expresses

a hydroxyphenylpyruvate dioxygenase (HPPD).

9. Chimeric gene according to claim 8, characterized in that the coding sequence is of bacterial or of plant origin.

10. Chimeric gene according to claim 9, characterized in that the coding sequence is from *Pseudomonas sp*.

11. Chimeric gene according to claim 8, characterized in that the coding sequence is a sequence such as defined in any one of claims 1 to 7.

12. Chimeric gene according to any one of claims 9 to 11, characterized in that the promoter regulation sequence favours the overexpression of the coding sequence.

13. Chimeric gene according to claim 12, characterized in that the promoter regulation sequence comprises at least one histone promoter.

14. Chimeric gene according to any one of claims 9 to 13, characterized in that it comprises a transit peptide between the promoter regulation sequence and the coding sequence.

15. Chimeric gene according to any one of claims 9 to 14, characterized in that it comprises, between the promoter regulation sequence and the coding sequence, an optimized transit peptide comprising, in the transcription sense, a sequence coding for a transit peptide of a plant gene coding for an enzyme with plastid localization, a part of the sequence of the N-terminal mature part of a plant gene coding for an enzyme with plastid localization, then a sequence coding for a second transit peptide of a plant gene coding for an enzyme with plastid localization.

16. Chimeric gene according to any one of claims 9 to 15, characterized in that it comprises a sequence of a transcription activator (enhancer) between the promoter regulation sequence and the coding sequence.

17. Vector utilizable for the genetic transformation of plants, characterized in that it comprises a chimeric gene according to any one of claims 9 to 16.

18. Plant cell, characterized in that it comprises a chimeric gene according to any one of claims 9 to 16.

19. Plant, characterized in that it is regenerated from cells according to claim 18.

20. Plant according to claim 19, characterized in that it belongs to the dicotyledonous plant family.

21. Plant according to claim 20, characterized in that it is a tobacco plant.

22. Plant according to claim 21, characterized in that it is tobacco PBD6.

23. Process of transformation of plants to make them tolerant to inhibitors of HPPD, characterized in that a gene expressing an exogenous HPPD is introduced into a vegetable cell.

24. Process according to claim 23, characterized in that the transfer is carried out with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*.

25. Process according to claim 23, characterized in that the transfer is carried out by delivery by bombardment with the aid of charged particles of the DNA.

26. Process of transformation of plants, characterized in that a gene expressing an exogenous HPPD is introduced into the vegetable cell as a selection marker.

27. Process according to any one of claims 23 to 26, characterized in that a chimeric gene according to any one of claims 8 to 16 is introduced.

28. Process of selective herbicide treatment of plants, characterized in that an HPPD inhibitor is applied to a transformed plant comprising cells according to claim 18.

29. Process according to claim 28, characterized in that the plants are the plants according to any one of claims 19 to 22.

30. Process according to any one of claims 28 and 29, characterized in that the plants are obtained by the process according to claims 23 to 27.

31. Process according to any one of claims 28 to 30, characterized in that the HPPD inhibitor is an isoxazole, a diketonitrile, a triketone or sulcotrione.

32. Process according to claim 31, characterized in that the isoxazole is 4-[4-CF_3-2-(methylsulphonyl)benzoyl]-5-cyclopropylisoxazole.

33. Sequence according to any one of claims 1 to 7, substantially as described herein with reference to the Figures and/or Examples.

34. Chimeric gene according to any one of claims 8 to 16, substantially as described herein with reference to the Figures and/or Examples.

35. Vector according to claim 17, substantially as described herein with reference to the Figures and/or Examples.

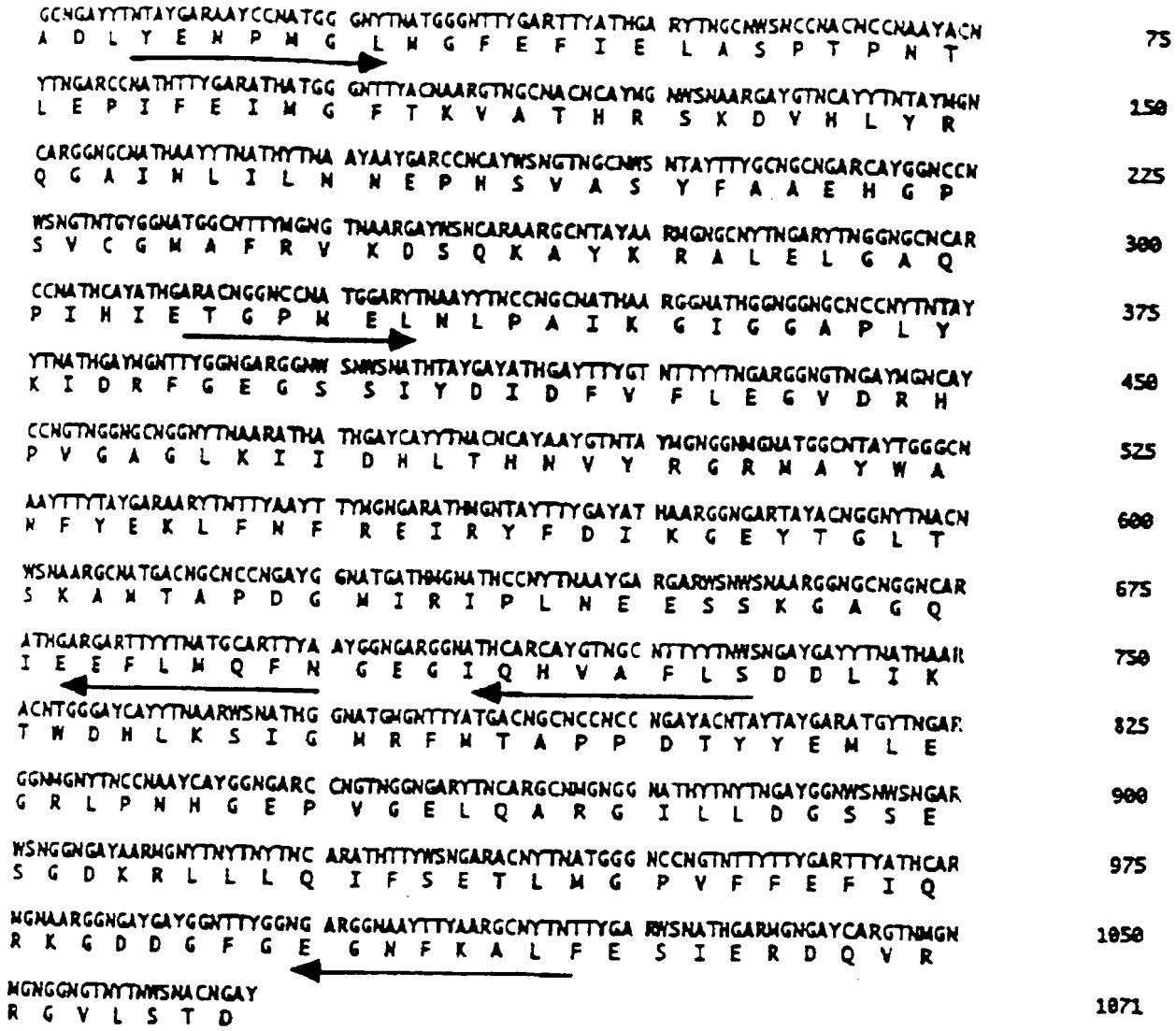
36. Plant cell according to claim 18, substantially as described herein with reference to the Figures and/or Examples.

37. Plant according to any one of claims 19 to 22, substantially as described herein with reference to the Figures and/or Examples.

38. Process according to any one of claims 23 to 32, substantially as described herein with reference to the Figures and/or Examples.

DATED this FIRST day of MARCH 2000
Rhone-Poulenc Agro

By DAVIES COLLISON CAVE
Patent Attorneys for the Applicants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675
ATHGARGARTTYYTNATGCARTTYA AYGGNGARGGNATHCARCAYGTNGC NTTYYTNWSNGAYGAYYTNATHAAR
I E E F L M Q F N G E G I Q H V A F L S D D L I K
750
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T W D H L K S I G M R F M T A P P D T Y Y E M L E
825
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G R L P N H G E P V G E L Q A R G I L L D G S S E
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975
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1071

Fig 1

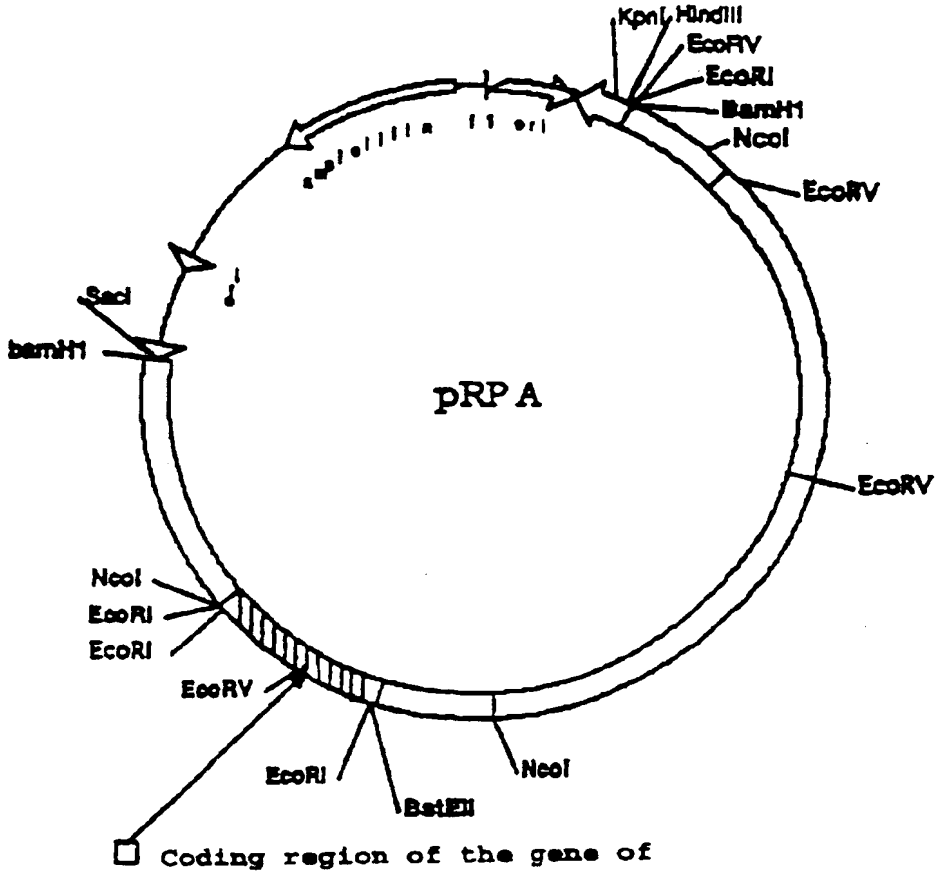
KpnI
HindIII
EcoRV
EcoRI
BamH1
NcoI
EcoRV
SacI
bamH1
pRP A
EcoRV
NcoI
EcoRI
EcoRI
EcoRV
EcoRI
NcoI
BstEII
Coding region of the gene of

Fig 2.

Consensus	.ADLYENPWG	LWGFEFIE.A	SPTP.TLEPI	FEIWGFTKVA	THRSK.VHLY	50
P. fluorescens	M.....	.....F.	....G.....	.....	.....N....	50
Pseudomonas sp.	-.....	.....L.	....N.....	.....	.....D....	49
Consensus	RQG.INLILN	NEP.S.ASYF	AAEHGPSVCG	WAFRVKDSQK	AY.RALELGA	100
P. fluorescens	...E.....	...N.I....	.....	.....	..N.....	100
Pseudomonas sp.	...A.....	...H.V....	.....	.....	..K.....	99
Consensus	QPIHI.TGPM	ELNLPAIKGI	GGAPLYLIDR	FGEGSSIYDI	DFV.LEGV.R	150
P. fluorescens	.....D....	.....	.....	.....	...Y....E.	150
Pseudomonas sp.	.....E....	.....	.....	.....	...F....D.	149
Consensus	.PVGAGLK.I	DHLTHNVYRG	RN.YWANFYE	KLFNFRE.RY	FDIKGEYTGL	200
P. fluorescens	N.....V.	.....	..V.....	.....A..	.....	200
Pseudomonas sp.	H.....I.	.....	..A.....	.....I..	.....	199
Consensus	TSKAW.APDG	WIRIPLNEES	SKGAGQIEEF	LWQFNGEGIQ	HVAFL.DDL.	250
P. fluorescens	.....S....	.....	.....	.....	.....T...V	250
Pseudomonas sp.	.....T....	.....	.....	.....	.....S...I	249
Consensus	KTWD.LK.IG	WRFWTAPPDT	YYEWLEGRLP	.HGEPV..LQ	ARGILLDGSS	300
P. fluorescens	....A..K..	.....	.....	D.....DQ..	.....	300
Pseudomonas sp.	....H..S..	.....	.....	N.....GE..	.....	299
Consensus	..GDKRLLLQ	IFSETLNGPV	FFEFIQRKGD	DGFGEGNFKA	LFESIERDQV	350
P. fluorescens	VE.....	.....	.....	.....	.....	350
Pseudomonas sp.	ES.....	.....	.....	.....	.....	349
Consensus	RRGVL..D					358
P. fluorescens	.....TA.					358
Pseudomonas sp.	.....ST.					357

Fig. 3